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Short Peptide-Based Tolerogens Without Self-Antigenic or Pathogenic Activity Reverse Autoimmune Disease[1]

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An immunodominant epitope of myelin basic protein (MBP), VHFFKNIVTPRTP (p87–99), is a major target of T cells in brain lesions of multiple sclerosis (MS), and this peptide can trigger experimental autoimmune encephalomyelitis (EAE). We designed truncated peptides based on this pathogenic 13-mer that are not antigenic. These short peptides reduced production of IFN-γ and TNF-α in vivo. Moreover, paraplegic rats given the 7-mer FKNIVTP in soluble form showed total reversal of paralysis in 24 h. Truncated peptides that are too small to stimulate antigenic responses to pathogenic regions of myelin basic protein are nevertheless effective tolerogens and are able to anergize autoreactive T cells. Short peptide-based tolerogens, devoid of immunogenic and pathogenic potential, may be attractive for therapy of autoimmune diseases. *The Journal of Immunology*, **1998, 160: 5188–5194.**

In autoimmune conditions, T cells reactive to self Ags escape elimination in the thymus and are activated in the periphery, where they can provoke damage in specific organs (1, 2). Restoration of self tolerance, without suppressing the immune system nonspecifically or stimulating further autoimmune reactions inadvertently, is a major challenge. Traditionally, protein molecules that are immunogenic are rendered tolerogenic by injecting them in incomplete Freund's or in soluble form. We have analyzed experimental allergic encephalomyelitis (EAE),[3] a particular version of a model of autoimmune disease that is induced in Lewis rats and that bears many similarities to the human disease multiple sclerosis (MS). T cells found in brain lesions of MS patients have TCR junctional rearrangements that are identical to T cells found in the spinal cords of Lewis rats immunized with a peptide of MBP, p87–99 (1–6). In addition a major T and B cell response in MS patients, particularly those of the HLA DR2 haplotype, is directed to MBPp87–99 (3–6).

We have previously demonstrated that the native peptide epitope MBPp87–99 induces EAE when given in CFA but reverses the disease when immunized in a soluble form (7). An altered version of MBPp87–99 (K > A) is not encephalitogenic when given in adjuvant and is able to reverse ongoing EAE when given in soluble form. Moreover, this altered peptide reduces production of TNF-α and IFN-γ in the lymph nodes of rats immunized with native MBPp87–99. MBPp87–99 (K > A) represents an altered peptide ligand at a major TCR binding contact. We have determined that the putative MHC binding sites for MBPp87–99 are F90, N92, I93, and V94, whereas the putative TCR contacts are K91, T95, and P96 (7). We now report the minimal structural requirements for a peptide that would tolerize animals with ongoing autoimmune disease. A panel of truncated and alanine substituted variants of p87–99 was constructed to demonstrate the role of each residue within the epitope in the induction of tolerance and to determine the minimal length of a peptide that could induce tolerance.

Materials and Methods

Induction of EAE and scoring

MBPp87–99 and various N terminus, C terminus, and double side truncated MBP peptides were dissolved in PBS at a concentration of 1 mg/ml and emulsified with an equal volume of incomplete Freund's adjuvant supplemented with 4 mg/ml heat-killed *Mycobacterium tuberculosis* H37Ra in oil (Difco Laboratories, Detroit, MI). Lewis rats were immunized s.c. in the hind foot pads with 0.1 ml of the emulsion and were monitored for clinical signs daily by an observer blind to the treatment protocol. EAE was scored as follows: 0, clinically normal; 1, flaccid tail; 2, hind limb paralysis; 3, front and hind limb paralysis.

Induction of passively transferred EAE

Transferred EAE was established by immunizing 6-wk-old female Lewis rats with 10^7 activated LS7–99 line cells (day 0). Five days later, at the onset of disease, sick rats were randomly distributed into different groups that were, or were not, injected i.p. 2 mg peptide in 1 ml PBS) with either native p87–99, OVA, various N terminus or C terminus truncated analogs or with N- and C-terminal truncations. Control rats were injected with PBS alone.

Lymphocyte proliferation assays

Proliferation of the MBPp87–99-specific CD4$^+$ line cells (LS7–99) to various C terminus and N terminus truncated variants of MBPp87–99 was determined in a proliferation assay as described elsewhere (7). Resting (6 days after last stimulation) LS7–99 cells were suspended in stimulation

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[3] Abbreviations used in this paper: EAE, experimental autoimmune encephalomyelitis; MBP, myelin basic protein; MS, multiple sclerosis; PE, pseudomonas; IC_{50}, concentration that inhibits 50%; SI, stimulation index.

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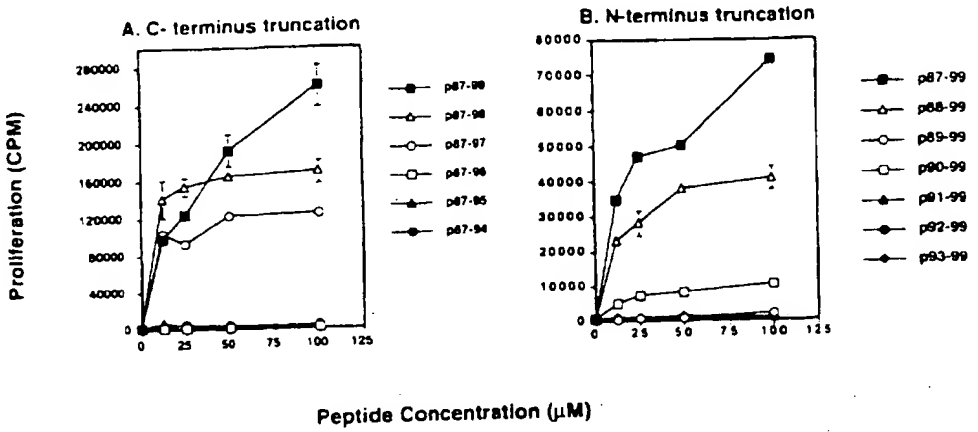


FIGURE 1. Proliferation of MBPp87–99-specific CD4$^+$ line cells to various C and N terminus truncated variants of the peptide. Proliferation of the MBPp87–99-specific CD4$^+$ line cells (L87-99) to various C terminus and N terminus truncated variants of MBPp87–99 was determined.

medium containing DMEM (Life Technologies, Gaithersburg, MD) supplemented with 2-ME (5×10^{-5} M), L-glutamine (2 mM), sodium pyruvate (1 mM), penicillin (100 U/ml), streptomycin (100 μg/ml), and 1% autologous Lewis rat serum. Cells were cultured for 72 h in U-shape 96-well microculture plates for 72 h at 37°C in humidified air containing 6.5% CO_2 at a concentration of 3×10^4 cells/well with 10^6 irradiated (2500 rad) thymocytes as accessory cells and various concentrations between 0 and 100 μM of different C terminus (Fig. 1A) or N terminus (Fig. 1B) truncated analogs of MBPp87–99. Each well was pulsed with 2 μCi of [^{3}H]thymidine (sp. act. 10 Ci/mmol) for the final 6 h. The cultures were then harvested on fiberglass filters, and the proliferative response was expressed as cpm ± SE.

Cytokine assays

Production of TNF-α and IFN-γ was measured in spleen cell cultures (Fig. 2, C and D) as follows. Spleen cells (10^7/ml) from each of the groups described above were cultured (1 ml per well) in 24-well flat-bottom tissue culture plates with, or without, 200 μM MBPp87–99. Twenty four hours and 48 h later supernatants were evaluated for levels of TNF-α and IFN-γ using available ELISA kits as follows: IFN-γ, Life Technologies rat IFN-γ kit, and TNF-α, Genzyme (Cambridge, MA) mouse cross-reactive with rat ELISA kit. Rat TNF-α served as a standard (PharMingen, San Diego, CA). The kits were used according to manufacturer's instructions. Levels of IL-4 were detected using a modification of an ELISA kit that we have established using a mouse anti rat IL-4 mAb (24050D OX-81; PharMingen) as a capture Ab and rabbit anti rat IL-4 biotin-conjugated polyclonal Ab (2411–2D; PharMingen) as a second Ab. Recombinant rat IL-4 was purchased from R & D (504-RL; R&D Systems, Minneapolis, MN). Goat anti rat IL-4 polyclonal Ab (AF-504-NA; R&D) was used as a neutralizing Ab. Spleen cells from each of the groups described above (Fig. 2A) were stimulated (96-well U-shape, 2.5×10^5 cells/well) for 72 h with or without various concentrations of MBP, MBPp87–99, and with or without the addition of rat IL-2 (50 U/ml, Becton Dickinson, San Jose, CA).

Measurement of cytosolic Ca^{2+}

Cytosolic Ca^{2+} was determined as follows. Resting (10 days after last stimulation) L87 cells were loaded with fura 2-AM at a final concentration of 5 μM in stimulation medium for 40 min at room temperature (24°C–25°C). Extracellular fura 2 was removed by washing twice with the incubating medium. Stained L87 cells (10^5 per well) were plated together with nonstained spleen cells (5×10^5 per well) in 96-well U-shape microtiter plates. The native peptide (MBPp87–99) and the double truncated variants p91–96 and p90–96 were each added at a final concentration of 10 μM to different wells and incubated for 5 min (37°C, 6.5% CO_2). The cells were then transferred to a nonfluorescent chamber mounted on the stage of an inverted microscope (Nikon, Japan). Fura 2 fluorescence was immediately measured using a dual wavelength system (Delta scan, PTI, Photon Technology International, South Brunswick, NJ). Briefly, light emitted from a Xenon arc lamp was led in parallel into two independent monochrometers to obtain quasi-monochromatic light beams of two different wavelengths, 340 and 380 nm. The two separate monochromator outputs were collected at the end of a bifurcated quartz fiber optic bundle. The emitted fluorescence (510 nm) was detected with a photomultiplier tube (710 PTM). All records are presented as a fluorescence ratio (R = F340/F380). A ratio of less than 1 indicates low cytosolic Ca^{2+}.

MHC binding assay

The ability of each of the N terminus truncated peptides at different concentrations from 0 to 200 μM to inhibit the binding of 10 μM of biotin-labeled native peptide was measured exactly as we described in detail elsewhere (7). The binding of p87–99 analogs to splenic adherent cells was measured by use of a fluorescence assay as follows. An amount equal to 5×10^5 splenic adherent cells in staining buffer containing 0.1% BSA in PBS was mixed with different concentrations of p87–99 analogs in individual wells of U-shape 96-well microculture plates and incubated at 37°C in a 6.5% CO_2 incubator. One hour later, biotin-labeled p87–99 (10 μmol) was added to culture wells for 4 h. The cells were washed three times with the staining buffer before PE-streptavidin (Becton Dickinson) was added as a second-step reagent (10 μl/well, 20 min) along with labeled mAb reacting with rat MHC class II I-A (0.4 μl/well, OX-6; PharMingen). The cells were washed twice before cytofluorographic analysis on a FACScan (Becton Dickinson). The fluorescence intensity was calculated by subtracting the fluorescence obtained from OX-6-positive cells stained with PE-streptavidin only from the fluorescence obtained with biotin-labeled p87–99 plus PE-streptavidin. The percentage of inhibition was calculated and is presented as IC_{50} values or as the percentage inhibition of mean relative binding.

Results and Discussion

The panel of truncated peptides was analyzed for a variety of in vivo and in vitro functions. Peptides were tested for their ability to induce active EAE when immunized in adjuvant, for their capacity to initiate a proliferative response in an encephalitogenic T cell line, L87–99, and for their ability to reverse ongoing paralysis, when inoculated in a soluble form. After truncation of V87, p88–99 was able to induce active EAE, stimulate L87–99, and reverse ongoing EAE (Table I (N terminus truncation); Table II (N terminus truncation, group c; and Fig. 1B)). In contrast, truncation of H88 resulted in a nonencephalitogenic peptide (Table I (N terminus truncation), 0/6 vs 6/6; $p < 0.01$) incapable of stimulating L87–99 (Fig. 1, 50030 ± 1100 vs 615 ± 220 cpm ± SE with a background of 314 cpm in response to 50 μM peptide; $p < 0.0001$). This peptide, p89–99, could reverse EAE (Table II (N terminus truncation, group d compared with a, 0/12 vs 12/12); $p < 0.005$). Further truncation at F89 maintained these features, since it was incapable of inducing EAE, did not stimulate L87–99, and could reverse ongoing EAE (Table II (N terminus truncation, group e compared with a, 0/12 vs 12/12); $p < 0.005$). In contrast, truncation of the F90, a critical MHC binding contact, abolished

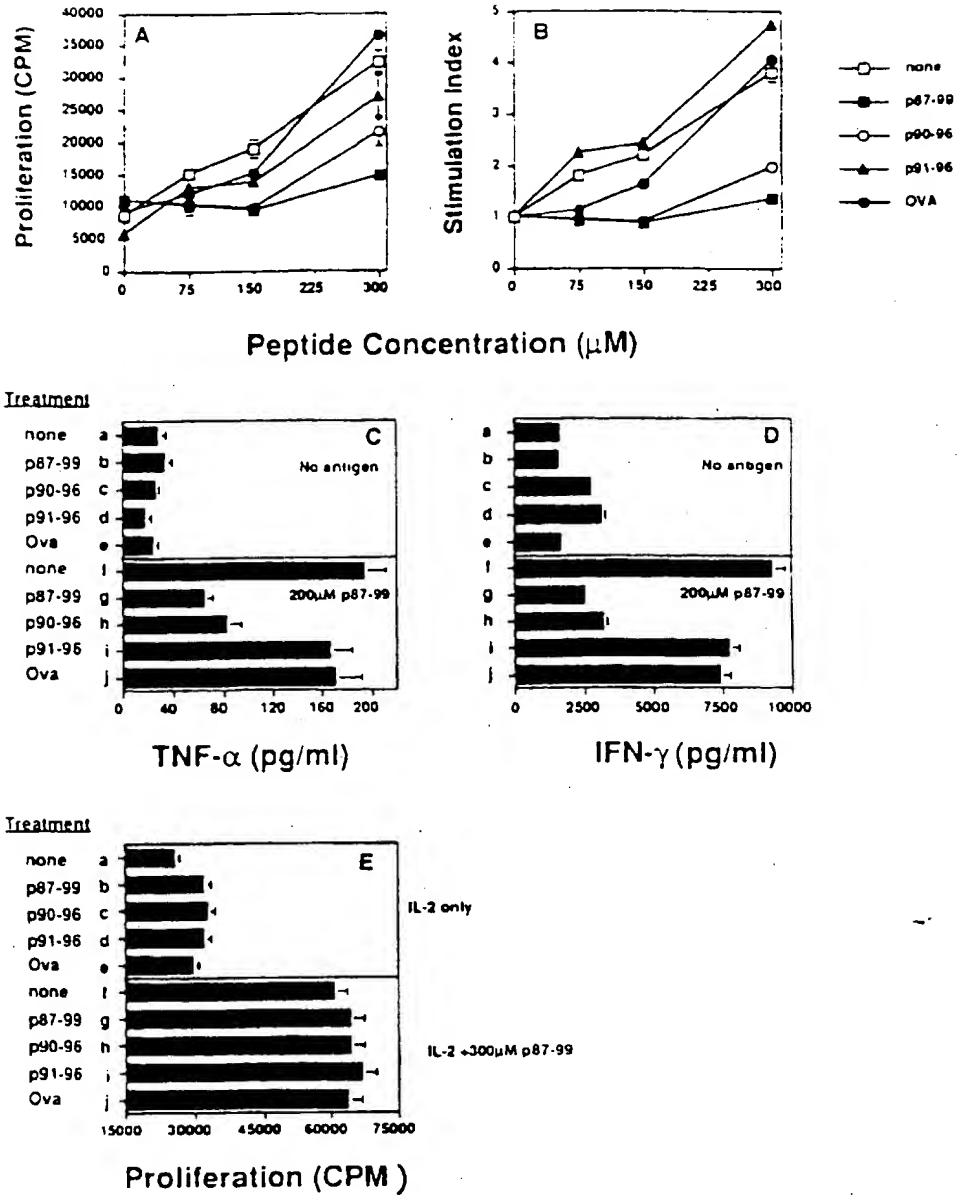


FIGURE 2. Soluble peptide therapy with a nonencephalitogenic 7-mer induces T cell unresponsiveness accompanied with a reduction in proinflammatory cytokine production. Five groups of rats were inoculated with 10×10^6 activated L87-99 cells to induce EAE. Four days later each of these groups was inoculated (i.p.) with either MBPp87-99 (■), p90-96 (○), p91-96 (▲), OVA (●), or with PBS alone (□). One day later the proliferative response in recipient spleen cells against the native peptide was measured (Fig. 2, *A* and *B*) as follows. Spleen cells were suspended in stimulation medium and cultured for 72 h in U-shape 96-well plates at a concentration of 2.5×10^5 cells/well together with various concentrations (0–300 μM) of the native peptide. Each well was pulsed with 2 μCi of [^{3}H]thymidine for the final 6 h as described above (Fig. 1). The proliferative response was expressed as cpm ± SE (Fig. 2*A*), or stimulation index (Fig. 2*B*). Production of TNF-α and IFN-γ was measured in these spleen cell cultures (Fig. 2, *C* and *D*, respectively). The proliferative response (cpm ± SE) of each group to 300 μM MBPp87-99 is shown in Fig. 2*E*.

the capacity of this peptide to reverse EAE. This finding suggests that binding of the tolerogen to MHC is necessary for effective induction of tolerance.

Analysis of C-terminal truncations showed that truncation of R97 abolished the ability of the peptide to induce EAE (Table I, 0/6 vs 6/6, $p < 0.01$) and to stimulate a proliferative response in L87-99 (Fig. 1*A*, 191500 ± 17100 vs 7000 ± 250 with a background of 300 in response to 50 μM peptide, $p < 0.0001$, accordingly). Nevertheless, p87-96 reversed EAE in all recipients within 24 h of treatment (Table II (C terminus truncation, group e compared with group a, 0/11 vs 11/11; $p < 0.005$). Further truncation of the proline at position 96 diminished the capacity of the peptide to reverse EAE (Table II (C terminus truncation, group f)).

A set of peptides with double truncations from the N and C termini were constructed to determine the minimal peptide capable of reversing EAE. FKNIVTP (p90-96) reversed ongoing EAE. All paralyzed rats treated with p90-96 went into total remission within 24 to 36 h, while PBS-treated control rats continued to develop

Table I *Induction of active EAE with various p87-99 truncated peptides*

Peptide	Sequence	IC_{50}* (μM)	L87-99[a] Proliferative Response (100 μM p87-99) (SI)	Active[c] EAE Incidence	First day of onset	Duration	Mean max. score
C terminus truncation							
p87–99	VHFFKNIVTPRTP	7	+ + + + +	6/6	12	7	1.5 ± 0.2
p87–98	VHFFKNIVTPRT	12	+ + +	6/6	12	7	1.5 ± 0.2
p87–97	VHFFKNIVTPR	11	+ + +	6/6	12	7	1.2 ± 0.1
p87–96	VHFFKNIVTP	81	−	0/6	−	0	0
p87–95	VHFFKNIVT	>200	−	0/6	−	0	0
p87–94	VHFFKNIV	>200	−	0/6	−	0	0
N terminus truncation							
p88–99	HFFKNIVTPRTP	7	+ + + + +	6/6	12	7	1.85 ± 0.3
p89–99	FFKNIVTPRTP	168	+ + +	0/6	−	0	0
p90–99	FKNIVTPRTP	49	+	0/6	−	0	0
p91–99	KNIVTPRTP	5	−	0/6	−	0	0
p92–99	NIVTPRTP	20	−	0/6	−	0	0
p93–99	IVTPRTP	>200	−	0/6	−	0	0
Double side truncations							
p90–96	FKNIVTP	>200	−	0/6	−	0	0
p91–96	KNIVTP	>200	−	0/6	−	0	0

* IC_{50} values were calculated as described in *Materials and Methods* and in Reference 7.

[a] The proliferative response of line L87-99 to its native peptide as well as to all the truncated peptides. Table I summarizes these results as follows: − = S.I. < 20, + = 20 < S.I. < 30, + + = 30 < S.I. < 40, + + + = 40 < S.I. < 50, + + + + = 50 < S.I. < 75, + + + + + = 75 < S.I. < 85, + + + + + > + + + + > + + + > + + > + > −, $p < 0.0001$

[c] Rats were immunized with p87–99/CFA or truncated peptides in CFA. EAE was monitored daily as follows: score 1 = paraplegia of the tail, score 2 = paralysis of hind limbs, score 3 = paralysis of hind + front limbs

hind limb paralysis that persisted for another 4 to 5 days (Table II (N + C terminus truncation, group b vs a, 0/10 compared with 10/10; $p < 0.001$). Truncation of the F90, a major MHC contact, abrogates the ability of the peptide to reverse EAE. This reinforces the idea that an effective tolerogen must be capable of binding to the MHC.

We next analyzed the mechanisms underlying the rapid reversal of disease, to see whether immunologic tolerance to the pathogenic

Table II. *Treatment of transferred EAE by soluble truncated analogs*[a]

Group Soluble Peptide (2 mg/ml)	EAE Incidence Day 5	Day 6–7	Day 8	Day 9
C terminus truncation				
a none	11/11	11/11	11/11	5/11
b p87–99	11/11	0/11	0/11	0/11
c p87–98	11/11	0/11	0/11	0/11
d p87–97	11/11	0/11	0/11	0/11
e p87–96	11/11	0/11	0/11	0/11
f p87–95	11/11	7/11	7/11	3/11
g p87–94	11/11	11/11	11/11	4/11
N terminus truncation				
a none	12/12	12/12	12/12	6/12
b p87–99	12/12	0/12	0/12	0/12
c p88–99	12/12	0/12	0/12	0/12
d p89–99	12/12	0/12	0/12	0/12
e p90–99	12/12	0/12	0/12	0/12
f p91–99	12/12	10/12	10/12	5/12
g p92–99	12/12	12/12	12/12	7/12
N− C terminus truncation				
a none	10/10	10/10	10/10	3/10
b p90–96	10/10	0/10	0/10	0/10
c p91–96	10/10	10/10	10/10	4/10
Single alanine substituted analogs				
a none	6/6	6/6	6/6	2/6
b p87–99	6/6	0/6	0/6	0/6
c p87–99 [90F>A]	6/6	6/6	6/6	0/6
d p87–99 [91K>A]	6/6	0/6	0/6	0/6
e p87–99 [92N>A]	6/6	1/6	1/6	0/6
f p87–99 [93I>A]	6/6	0/6	0/6	0/6
g p87–99 [94V>A]	6/6	1/6	1/6	0/6
h p87–99 [95T>A]	6/6	6/6	6/6	0/6
i p87–99 [96P>A]	6/6	1/6	1/6	0/6

[a] Transferred EAE was established by immunizing 6-wk-old female Lewis rats with 10^7 activated L87-99 line cells (day 0). Five days later, at the onset of disease, sick rats were randomly distributed into different groups that were, or were not, injected i.p. 2 mg peptide in 1 ml PBS+ with either native p87–99, OVA, various N terminus or C terminus truncated analogs, or with N- and C-terminal truncations. Control rats were immunized with PBS alone.

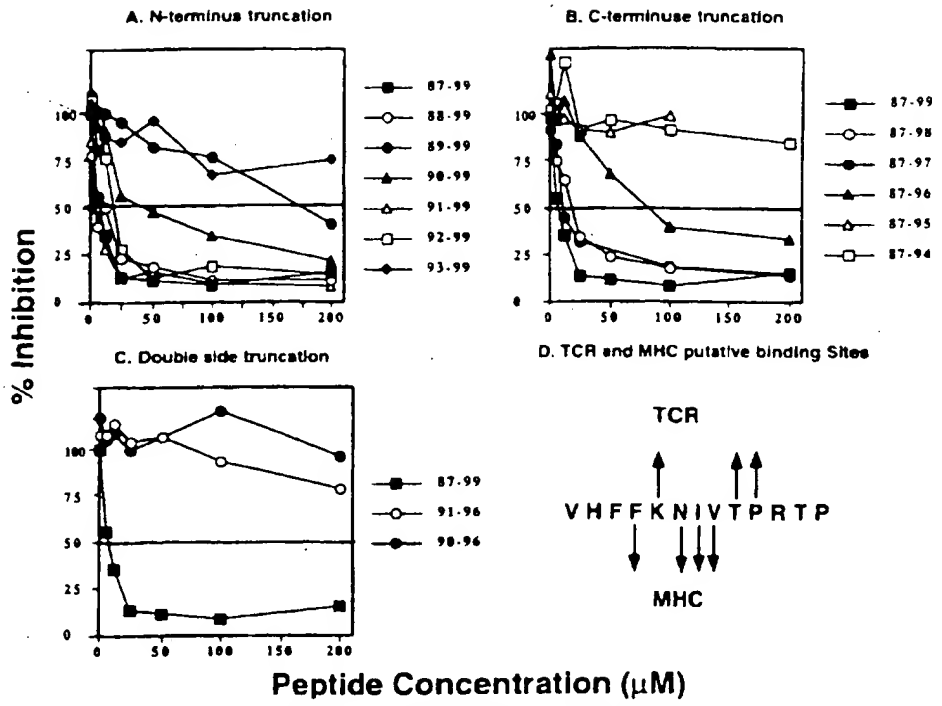


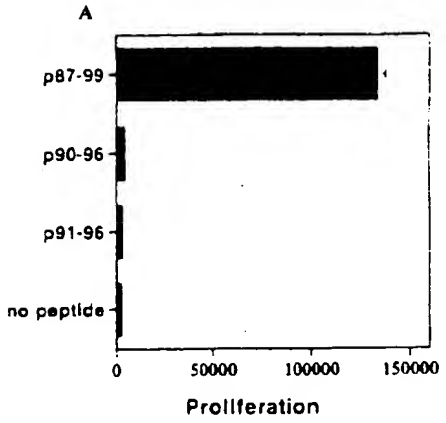
FIGURE 3. MHC binding competition between MBPp87–99 truncated analogs and MBPp87–99. The ability of each of the N terminus truncated peptides at different concentrations from 0 to 200 μM to inhibit the binding of 10 μM of biotin-labeled native peptide was measured exactly as we described in detail elsewhere (7). Data are presented as the percentage inhibition of mean relative binding. The IC_{50} values are shown in Table I.

peptide MBPp87–99 was induced. Five groups of rats were inoculated with 10×10^6 activated L87–99 cells to induce EAE. Four days later each of these groups was inoculated (i.p.) with either p87–99, p90–96, p91–96, OVA; or with PBS alone (Fig. 2). One day later the proliferative response in recipient spleen cells against the native peptide was measured (Fig. 2, *A* and *B*). Rats inoculated either with the native peptide or with p90–96 developed a substantially reduced response against the native peptide compared with those seen in spleens from rats immunized with OVA, p91–96, or with PBS alone (Fig. 2, *A* and *2B*; SI = 1.4 and SI = 2.0 compared with SI = 4.0, SI = 4.7, and SI = 3.8 in response to 300 μM of the native peptide, backgrounds 10940, 10950, 9080, 5700, and 9080, respectively; $p < 0.005$). Production of TNF-α and IFN-γ was measured in these spleen cell cultures (Fig. 2, *C* and *D*). Spleen cells from rats immunized with the protective peptides p87–99 or p90–96, but not with the nonprotective p91–96, OVA, or PBS alone produced reduced amounts of TNF-α and IFN-γ (for TNF-α, 64 ± 8 and 82 ± 12 pg/ml compared with 166 ± 18, 70 ± 22, and 194 ± 18 pg/ml in response to 200 μM peptide, $p < 0.001$, Fig. 2*C*; for IFN-γ, 2500 ± 880 and 3190 ± 320 pg/ml compared with 7700 ± 870, 7380 ± 730, and 9270 ± 985 pg/ml in response to 200 μM peptide, $p < 0.001$, Fig. 2*D*). TNF-α and IFN-γ are two inflammatory cytokines that play a major role in the initiation and development of T cell mediated autoimmunity (8–12).

We investigated whether tolerance induction by either p87–99 or by the nonencephalitogenic p90–96 might be reconstituted by rat IL-2. Addition of rIL-2, in the presence of a high dose of the native peptide (300 μM), totally abrogated T cell tolerance (Fig. 2, *E* compared with *A* and *B*). Thus, anergy is induced by p90–96. Interestingly, only the combination of a high dose of Ag (300 μM but not 200 μM or 100 μM) and an elevated level of IL-2 (50 U/ml but not 10 U/ml) was sufficient to abrogate T cell tolerance (Fig. 2, *E* compared with *A* and *B*; data for 100 and 200 μM not shown). Conceivably, in vivo tolerized cells enter the programmed cell death pathway, which may be reversible only after addition of threshold levels of IL-2 plus antigenic stimuli.

The markedly reduced production of TNF-α and IFN-γ in spleen T cells from p90–96- or p87–99-tolerized rats, in response to 200 μM p87–99, was not accompanied by an increase in IL-4 production (28 ± 2.1 pg/ml and 25.2 ± 3.3 pg/ml in p90–96- and p87–99-tolerized rats vs 45 ± 3.4 and 40.2 ± 3.7 pg/ml in p91–96-tolerized and control rats). Consequently, anti-IL-4- neutralizing Abs, added at various concentrations between 150 and 450 ng/ml, were incapable of restoring the proliferative response of T cells from p90–96- or p87–99-tolerized rats (data not shown), suggesting that more than alteration of the Th1/Th2 balance toward Th2 response is involved in the induction and maintenance of the tolerant state in soluble peptide therapy.

We have further investigated the influence of alanine substitutions at each of the different amino acids within the 7-mer core FKNIVTP. For single alanine substitutions in p87–99, substitution of alanine for either K, N, I, V and P at positions 91, 92, 93, 94 and 96 had no effect on the ability of the peptide to reverse EAE (Table II; single alanine substituted analogs). Substitution of A for F at position 90, a major MHC binding residue, and the A for T at position 95 abolished the ability to reverse EAE (Table II, single alanine substituted analogs, 6/6 sick rats for 90F > A and 95T > A). We have recently demonstrated that K, T, and P at positions 91, 95 and 96 are putative TCR binding sites (Ref. 7 and Fig. 3). It is therefore remarkable that only one of these TCR binding sites, T at position 95, is essential for disease reversal. MBPp87–99 binds MHC via F, N, I, and V at positions 90, 92, 93, and 94 (7). Any single alanine substitution at any of these positions (Ref. 7 and Fig. 3) resulted in reduced MHC binding, compared with the



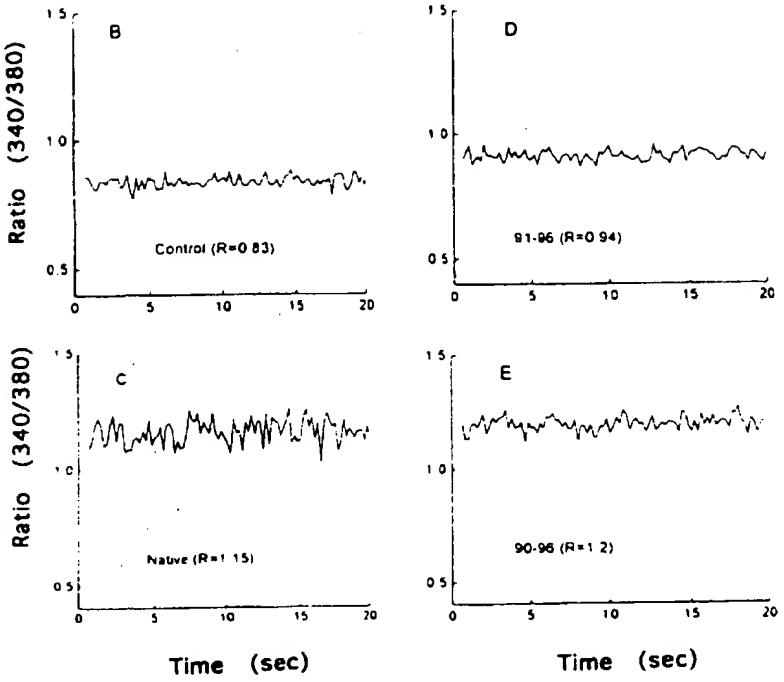


FIGURE 4. *A*, Proliferation assay of L87 to 10 μM of the following peptides: MBPp87–99, p90–96, and p91–96. The proliferation assay was performed as described above (Fig. 2, *A* and *B*). *B–E*, Elevation of cytosolic Ca^{2+} in representative L87 cells in response to the various peptides, on which proliferation was measured in Fig. 3*A*.

full-length peptide p87–99. Substitutions of A at 92N, 93I, and 94V were still capable of inducing EAE (7). Only the analog with a single substitution of F90 > A was unable to induce EAE (7). Concordant with this observation, this particular MHC binding site was critical for the induction of T cell tolerance and reversal of EAE (Table II, single alanine substituted analogs, group c, 6/6 sick rats).

To further investigate whether there was any association between affinity of MHC binding and the ability to reverse EAE, the IC_{50} values of all truncated analogs was determined in a competition assay on live APCs (Fig. 3). p90–96 reverses disease effectively, even though it is a weak MHC binder (Fig. 3 and Table I, IC_{50} > 200 vs IC_{50} = 7 for native p87–99; $p < 0.001$). This indicates that disease reversal may occur despite low MHC binding, provided that a sufficient number of MHC and TCR contacts are present.

The truncated peptide p90–96 induces T cell tolerance (Fig. 2) and reverses EAE (Table II, N + C terminus truncation). Truncation of F90 (p91–96) abolishes these capabilities when tested at a concentration of 10 μM (Fig. 4*A*, 134,000 ± 4,200 compared with 4,400 ± 570, 3,100 ± 550 in response to p90–96 and p91–96, respectively, with a background of 2,350 ± 440; $p < 0.001$). Similar results were seen at 100 μM (data not shown). This implies that tolerance induction and T cell activation are initiated by distinct amino acid residues within a T cell epitope.

In another experiment rats were immunized with p90–96 emulsified in CFA. Nine days later, the development of spleen T cells proliferative response against self and against the native peptide (p87–99) was recorded. Even though p90–96 induced a profound anti-self response (8540 ± 513 cpm with a background of 2080, SI = 4.1, in response to 100 μM of p90–96), it did not elicit any notable cross-reactive response against the native epitope (2340 ± 410 cpm with a background 2550 ± 230, in response to 100 μM of p90–96).

In a further experiment each of the above analogs and the native peptide were all compared for their abilities to initiate Ca^{2+} influx

in a T cell line recognizing MBPp87–99. The native peptide (Fig. 4*C*) induced Ca^{2+} flux, increasing the fluorescence ratio from 0.88 ± 0.02 in control cells (Fig. 4*B*; $n = 7$) to 1.02 ± 0.03 ($n = 18$), $p < 0.01$. Similarly, p90–96 also increases Ca^{2+} flux, (Fig. 4*E*, $n = 20$, 1.12 ± 0.04 vs 0.88 ± 0.02; $p < 0.01$). In contrast, p91–96 (Fig. 4*D*) had no significant effect on the level of cytosolic Ca^{2+} in the p87–99-reactive T cells ($R = 0.92 \pm 0.03$, $n = 19$). Taken together, these results suggest that T cell tolerance can be induced only by epitopes capable of triggering Ca^{2+}_i influx after TCR engagement.

The minimal requirement for recognition of an autoantigen requires only five native residues in a stretch of an 11-amino acid epitope (13). The minimal size for a peptide capable of inducing EAE is a 6-mer. Thus in H-2^u mice the 6-mer Ac1–6 of MBP produced EAE, albeit at a frequency of 10% from that induced with the optimal 11-mer peptide, Ac1–11 of MBP (14). Short altered peptides are advantageous because of their reduced pathogenicity compared with native peptides, as well as their inability to stimulate a cross-reaction with T cells that react to the native pathogenic epitope. These 7-mer peptides, in this study, represent the shortest tolerogens shown to date and emphasize the minimal requirements for in vivo induction of T cell tolerance. It is striking that only one MHC anchor and one TCR binding site are essential for initiation of Ag-specific T cell tolerance. Moreover, T cell tolerance was associated with the capacity of the tolerogen to initiate Ca^{2+} influx, implying that induction of Ag-specific irresponsiveness is an active process, as recently suggested by in vitro systems (15, 16). Interestingly, it has been recently demonstrated that, in B lymphocytes, the amplitude and duration of calcium signals control differential activation of proinflammatory transcriptional regulatory factors like NF-κB, c-Jun N-terminal kinase (JNK), and NF-AT (17, 18). Short, nonstimulatory peptides based on truncated epitopes of pathogenic peptides may therefore be practical for therapy of autoimmune disease.

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